STUDY OF AN ARS-LIKE FUNCTION OF MAP UNITS 88-100 OF PARVOVIRUS LUIII

By

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ABSTRACT

The synthesis of minus strand by parvovirus LuIII requires replication from the left palindrome of the plus strand DNA. An origin of replication yet to be identified must be present at the right end of the minus strand, required for the synthesis of plus strand. Parvovirus LuIII contains a unique A/T-rich sequence that shares a sequence homology with the autonomously replicating sequences (ARS) found in yeast. To test if the A/T rich region and sequences downstream the A/T sequence are responsible for autonomous replication, nucleotides 4527 to 5135 of LuIII were cloned (pUraLu88-100) into a vector containing the URA3 gene as an auxotrophic marker. pUra-Lu88-100 was transformed into *Saccharomyces cerevisiae* (SEY S288C,ura3-) by the electroporation method. Plasmid DNA was isolated from the resulting colonies and transformed into *Echerichia coli* DH5α cells. Analysis performed to the plasmid rescued from yeast indicate that pUra-Lu88-100 was capable of existing in a free state in *S. cerevisiae*, suggesting that the elements required for a limited ARS-like function are present in at the right end of the LuIII viral genome.

Resumen

La síntesis de la hebra negativa por el parvovirus LuIII requiere la replicación del palindrome izquierdo de la hebra positiva de ADN. Un origen de replicación que no a sido identificado debe estar presente en el extremo derecho de la hebra negativa. El parvovirus LuIII contiene una secuencia única rica en A/T que comparte homología con las secuencias de replicación autónoma encontradas en levaduras. Para evaluar si la región rica en A/T y las secuencias río abajo de ésta son responsables de replicación autónoma, los nucleotidos 4527 a 5135 de LuIII fueron clonados (pUraLu88-100) en un vector que contenía el gene URA3 como un marcador auxotrófico. pUra-Lu88-100 fue transformado en el *S. cerevisiae* (SEY S288C, ura3 -) por el método de electroporación. El ADN del plásmido fue aislado de las colonias resultantes y transformado posteriormente en células del *E. coli* DH5α. Análisis realizados al plásmido rescatado de la levadura indican que el clon pUra-Lu88-100 fue capaz de existir en un estado libre en *S. cerevisiae*, sugiriendo que los elementos requeridos para llevar a cabo replicación autónoma limitada similar a la descrita para ARS están presentes en el terminal derecho del genoma viral de LuIII.

Dedication

To you Mine, my angel, that from heaven, next to God, guides my steps. I miss you sister. "Mami", thanks for your unconditional love and support, you are my inspiration. To my son Jean Michael, baby you are my reason of being.

Acknowledgements

I would like to thank God for all the special people in my life, my family and my friends. Special thanks to Dr. Nanette Diffoot-Carlo, for being my mentor in this process, for teaching me the way to excellence and for believing in me and in my work. Thanks for all the professional and personal support and all the opportunities given to me. Special thanks to my boyfriend Jose Luis for all the love and support during these years. Thanks to taking care of Jean when I had to study and for loving him as much as I do. Very special thanks to my best friend Lisandra Vélez. God brought you into my life little sister. You taught me about the richness of our hearts. You are so brilliant.

Thanks to my other family who teaches me about true friendship and sisterhood, my laboratory companions. Omayra "organization", Alina "in fashion", Militza "affection", and Mildred "patience". We are more than partners, we are friends. Thanks to Nancy Arroyo who colaborated with me to in this work and taught me about faith and to Aixa Sánchez for your knowledge. Special thanks to Dr. Govind Nadathur to provide me the yeast plasmids used for this investigation. Thanks to Dr. John Gill and Dr. Arturo Massol for being part of my graduate comitte and for their confidence.

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CHAPTER I
Introduction

The *Parvoviridae* family consists of small, icosahedral, single stranded DNA viruses with linear genomes that have palindromic sequences at each end. Based on their replication strategy, these viruses can be divided into two main categories: dependent viruses, which require coinfection with a helper virus for replication, and autonomous viruses, which are replication-competent. The autonomously replicating parvoviruses can infect cells without integrating into the host genome; this characteristic along with having small genomes being of non-pathogenic nature makes these viruses suitable transient viral vectors for the delivery of genes that code for therapeutic proteins.

Parvovirus LuIII, first isolated from a human cell line is an autonomous replicating virus. The entire genome of LuIII has been sequenced and the results demonstrate that LuIII has the same genomic organization as the rodent parvoviruses. When the LuIII sequence is compared to that of the rodent parvoviruses, Minute Virus of Mice (MVM) and Hamster virus (H-1) share over 80% of sequence identity. Regardless of their sequence homology these viruses have noteworthy differences. MVM and H-1 encapsidate 99% minus strand, whereas LuIII encapsidates equal amounts of both the plus and minus DNA strands with equal frequency. These differences in the encapsidation pattern have been suggested to be related to the adenine-thymine rich region found at map unit 89 of the LuIII genome, six nucleotides downstream of the end of the right open reading frame (ORF). The A/T nature of this LuIII region (5'TATGCTCTATGCTTCATATATATTATAATATTATTATACTAACTAA-3')

resembles autonomous replicating sequences (ARS) found in yeast. ARS elements consist of A/T rich regions that contain discrete sites in which mutations have been shown to affect origin function. Given that the LuIII A/T rich sequence shares a sequence similarity with the ARS's found in yeast, Arroyo (3), examined the possible ARS function of this LuIII A/T sequence. She cloned exclusively the LuIII A/T rich sequence into a yeast clone lacking an ARS, and tested the replication activity of the resulting clone, pNA1, in *S.cerevisiae*. Subsequent transformations into *S.cerevisiae* resulted in microcolonies, suggesting at the LuIII A/T rich sequence could direct autonomous replication. The colonies obtained could not be subcultured. Arroyo suggests that the right terminal sequence of LuIII, where this A/T sequence is found, could contain other elements necessary to maintain the ARS function.

The purpose of this work is to continue the work described by Arroyo and determines if these elements are present in the sequences found across the right end (map units 88-100) of the parvovirus LuIII genome. A possible origin of replication at the right terminus of LuIII could explain the synthesis of the plus strand of this parvovirus.

CHAPTER II
Literature Review

The *Parvoviridae* are a large family of small viruses, which infect animal species that range from insects to humans (31, 83). Their single stranded genomes are approximately 5,000 bases in length. Since parvoviruses have no means to force the cell into S phase, they replicate in cells that are actively synthesizing their DNA (101). The *Parvoviridae* family is divided into two sub-families, the *Densovirinae* and *Parvovirinae* (Table 1). The *Densovirinae* can replicate in insect host cells and other arthropods (99). This group is divided into three genera, *Densovirus, Iteravirus* and *Contravirus*. The *Parvovirinae* sub-family is divided into genera, *Erythrovirus, Dependovirus and Parvovirus* (9).

Erythrovirus, comprises the human parvoviruses B19 and V9, and the simian parvovirus (SPV). B19 and V9 are the only parvoviruses that are know to cause a disease in humans (25). B19 is associated with the common childhood disease erythema infectiosum or fifth disease (1). Furthermore, acute and chronic polyarthralgia (67, 76) and arthritis are also (94, 96) frequently associated with B19 infections, particularly in adult women. The pathogenic mechanisms underlying leukopenias and arthritis are not known. Since replication of B19 virus is strongly restricted to the erythroid progenitor cells in the bone marrow, it is not clear how the virus causes destruction of other cells. Depending on the patient's hematological and immunological status, B19 infections can provoke a variety of additional diseases such as aplastic crisis and hydrops fetalis (72).

V9 was isolated from a child with transient aplastic anemia (46). Since this isolate is a representative of a new B19 variant, the taxonomic position of V9 remains unclear. Based on phylogenetic analysis, Lukashov et al. have suggested that the separation between B19 and V9 was probably an ancient event (54).

O'Sullivan et al. identified SPV (70). It was isolated from a cynomolgus monkeys *Macaca fascicularis* with severe anemia. SPV exhibits a high degree of homology with B19 and has also a predilection for erythroid cells (12). These similarities have led to the proposal that SPV along with B19 be classified as a member of the erythrovirus genus. The use of SPV as an animal model for human B19 parvovirus replication has been suggested (70).

Dependovirus, consists of 6 types of replication defective adeno-associated viruses (AAV1-6). AAV's are non-pathogenic human parvoviruses. At either end of the genome AAV has a 145 bp inverted terminal repeat which contains a promoter (ITR). The ITR's are the only *cis*-acting elements necessary for AAV integration, replication, encapsidation and chromosomal excision (64). AAV infects both proliferating and resting cells and is naturally replication deficient due to their dependence on the replication of the helper virus, adenovirus or herpesvirus. In the absence of a helper virus AAV can integrates with a high frequency at a specific location (19q 13-qter) into the host genome (10, 50).

Parvovirus genus includes the autonomous, host specific mammalian viruses. They are termed autonomous viruses because they have all the information and functions necessary to replicate in suitable host cells (9, 101). Replication of the autonomous parvoviruses takes place in the nucleus of the host cell. The critical

functions for virus replication are usually supplied during the S early G-2 phase of the cell cycle (31).

There are a variety of parvovirus sub-groups. Feline panleukopenia virus (FPV), mink enteris virus (MEV) and canine parvovirus (CPV) are classified within the feline parvovirus sub-group. Porcine parvovirus (PPV) is another member of the parvovirus genus. Minute virus of mice (MVM), H-1 and LuIII are classified as rodent parvovirus (9, 54). MVM was isolated from a stock of mouse adenovirus. H-1 was originally isolated as a contaminant of a human tumor cell line (Hep1) that had been passaged in rats laboratory. LuIII was isolated as a contaminant of a human lung carcinoma (9).

Rodent parvoviruses provide an excellent model system to study genome organization and virus-host cell interaction. These viruses may have potential applications when relatively transient gene transfer is desired, because they do not integrate into the host genome.

Table 1. Organization of the Parvoviridae Family (9).

PARVOVIRIDAE FAMILY			
Sub-family	Genus	Type Species	Host
Densovirinae	Densovirus	Junonia coenia densovirus	Invertebrates
	Iteravirus	Bombyx mori densovirus	
	Brevidensovirus	Aedes aegypti densovirus	
Parvovirinae	Erythrovirus	B19 virus, V9, SPV	Vertebrates
	Dependovirus	Adeno-associated virus	
	Parvovirus	Mice minute virus, H-1, LuIII	

Characteristic of Virion Morphology

Parvovirus particles are non-enveloped, have icosahedral symmetry and are very small in diameter (18-26 nm). Nucleocapsids are isometric, approximately 60 capsomers per nucleocapsid and appear to be round. The particles contain DNA and protein. Parvoviruses are one of the most resistant viruses known. Together, the three-capsid proteins VP1, VP2, and VP3 confer considerable stability to the virions, allowing for resistance to inactivation by pH, solvents such as alcohol and ether and temperatures as high as 50 °C (9).

Genome organization

The parvovirus genome is a linear, non-segmented, single stranded DNA strand of approximately 5,000 bases in length. This single stranded DNA can be of positive or negative (complementary to viral mRNA) polarity. The genome has palindromic sequences at their 3' and 5' termini that can fold back on themselves to form two "hairpin" or secondary structures that are stabilized by self-hydrogen bonding. These "hairpin" structures contain the *cis*-acting signals indispensable for genome replication (4, 86).

The viral genomes that have been characterized have 3' and 5' terminal hairpin sequences that are unrelated in sequence (MVM), or sequences that are closely related (AAV and B19). Adeno-associated viruses have an inverted terminal repetition of 147 bases long, and both of its termini are identical (52, 56). B19 also has identical terminal repeats (9).

The 3' terminus (left end of the minus strand) of the autonomous parvoviruses is 115 to 122 bases long, and can assume a rabbit ear (Y or T-shape) structure. The 5' terminus (right end of the minus strand) is 206 to 242 bases long and can assume a rabbit ear (Y or T-shape) or a hairpin (U-shape) structure (9, 82). These termini are not perfect palindromes. Internal unpaired nucleotides within the hairpin stem form a "bubble", with two distinguishable forms, "flip" and "flop" (87). The "bubble", consist of a triplet 5' -GAA- 3' on one strand opposed by a dinucleotide 5' -GA- 3' on the complementary strand. Although these arms have nearly identical sequences, only the arm containing the GA dinucleotide functions as a replication origin (28).

Parvovirus LuIII has non-identical imperfect termini. The left end (3') can assume a T-shape configuration and the right end (5') has a U-shape configuration (Figure 1 A) (34). The left end of LuIII is 122 bases long and can exist in the "flip" (GAA opposed to GA) conformation. The right end is 211 bases long and exists in the "flip" and "flop" (TTC opposed to TC) conformations (Figure 1 B).

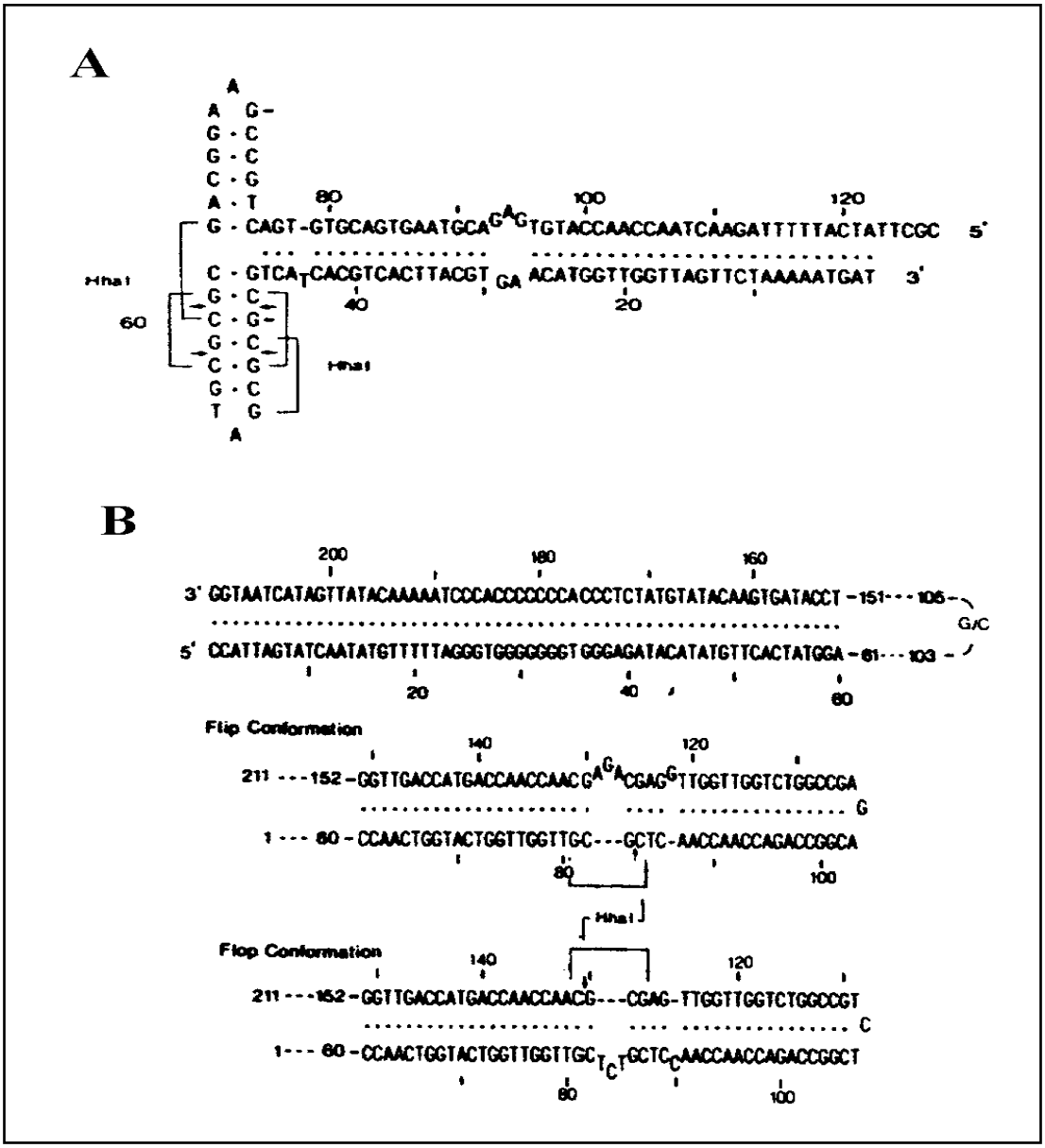


Figure 1. Palindromic Termini of Parvovirus LuIII. **A**, DNA sequence at the left terminus of the minus strand of LuIII, which can assume a T-shape structure. **B**, DNA sequence at the right terminus of the minus strand of LuIII. This terminus can assume an U-shaped structure, with "flip" and "flop" conformations (Reprinted from Diffoot et al; 1989).

Transcription

B19 initiates transcripts from a promoter at map unit 6 (9). AAV has three promoters located at map units 5, 19 and 40. By differential splicing at each location, AAV produces six capped and polyadenylated RNA's. The transcripts from promoters P5 and P19 encode the Rep proteins, called Rep 68, Rep 78, Rep 52 and Rep 40 (41). These proteins are equivalent to the non-structural proteins of other parvoviruses. Promoter P40 directs transcription of the AAV structural proteins, VP1-3 (9).

The autonomous parvovirus genome is organized into two major open reading frames (ORF's) under the control of two promoters, located at map units 4 (P4) and 38 (P38) (Figure 2) (21). The early P4 promoter controls the transcription of the non-structural proteins NS1 and NS2 (30). NS1 and NS2 are involved in replication and cytotoxicity (15, 28). NS1 is the most important non-structural protein, essential for productive replication in all cell types (66). It is involved in many processes during the virus cycle. It controls promoter activities (77), causes alteration of the cell physiology and morphology and is the initiator protein for viral DNA replication (15, 29, 69). It is a site-specific protein, recognizing the sequence $(ACCA)_{1-3}$ present in the viral origins (26). NS2 is an auxiliary regulatory protein, which contributes to cell-type specificity and host range pathogenicity. (66). NS1 also transactivates P38, thus allowing the synthesis of the capsid proteins, VP1 and VP-2 towards the end of the viral cycle (35). The molecular weight of VP-1 and VP-2 are approximately 83 and 64 kDa respectively (88). The VP-1 capsid protein is required for infection. Expression of VP-2 was found to be sufficient to generate capsids containing packaged DNA, but these particles

were non-infectious (102). Incorporation of VP-1 into the capsids containing VP-2 is essential for infectivity. The expression of VP-1 alone did not generate virus particles (93). An alteration in the N-terminal of VP-1 makes the capsid inefficient for cell infection (100). VP-3 is derived by protease cleavage of VP-2 and is the major protein containing the central structural motif of VP-2, this topology has been found in many other icosahedral viruses (9).

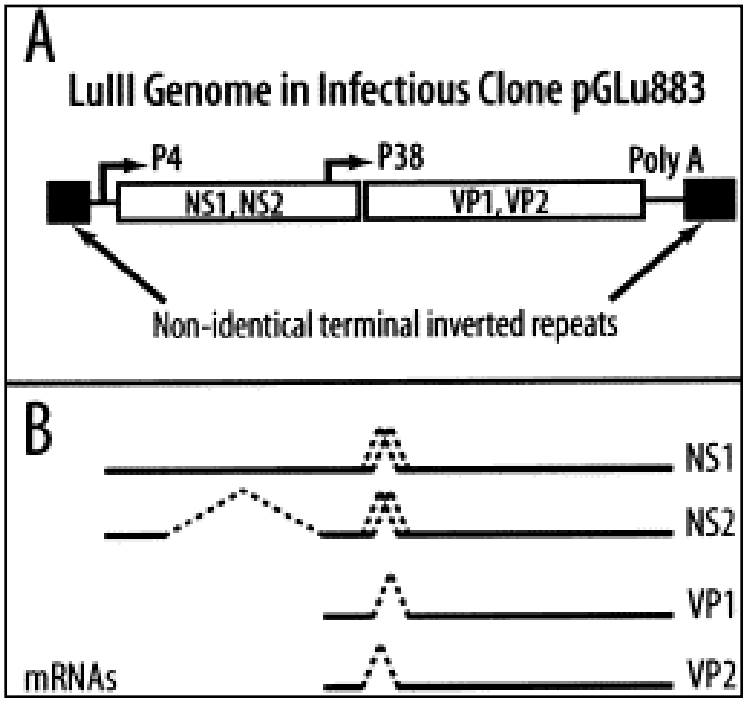


Figure 2. **Organization of the LuIII genome**. A, Genomic map of LuIII. B. Proposed transcription map for LuIII. NS; non-structural proteins, VP; capsid viral proteins (Reprinted from Maxwell et al; 2002).

Replication of autonomous parvoviruses

The replication and assembly of autonomous parvoviruses occurs in the nucleus and are highly dependent on cellular functions for their genome replication. (31, 89). It starts at the time of host cell entry into S-phase (31), indicating a close relation between host enzymes and cellular functions and viral DNA synthesis. Replication of single-stranded parvovirus proceeds through a series of monomeric and concatemeric duplex replicative-form (RF) intermediates by a unidirectional, leading-strand specific, cellular replication fork leading by the initiator protein NS1 (4, 26). In order to initiate DNA synthesis, the 3' end of the viral genome folds into a hairpin structure to serve as a primer for initiation of DNA synthesis by a cellular DNA polymerase (Figure 3). Elongation from the 3'OH of the hairpin allows to synthesize a complementary copy of the parental strand. Next, an obligatory covalently closed replicative form (cRF) is generated (step 1). The opening of the cRF is given by unpaired sequences, which serve, as cleavage sites for the NS1 nuclease. Subsequently, NS1 becomes covalently attached to the right termini of the viral genome, followed by initiation of displacement synthesis, copying of the right end sequence, and formation of a terminal extended molecule (5'eRF) (step2). Hairpin refolding at the extended terminus creates a structure called rabbit ear replicative form (5'reRF) (step3). The complement of the 5' terminal hairpin then serves as a primer for strand displacement synthesis and dimeric replicative form (dRF) (step 4).

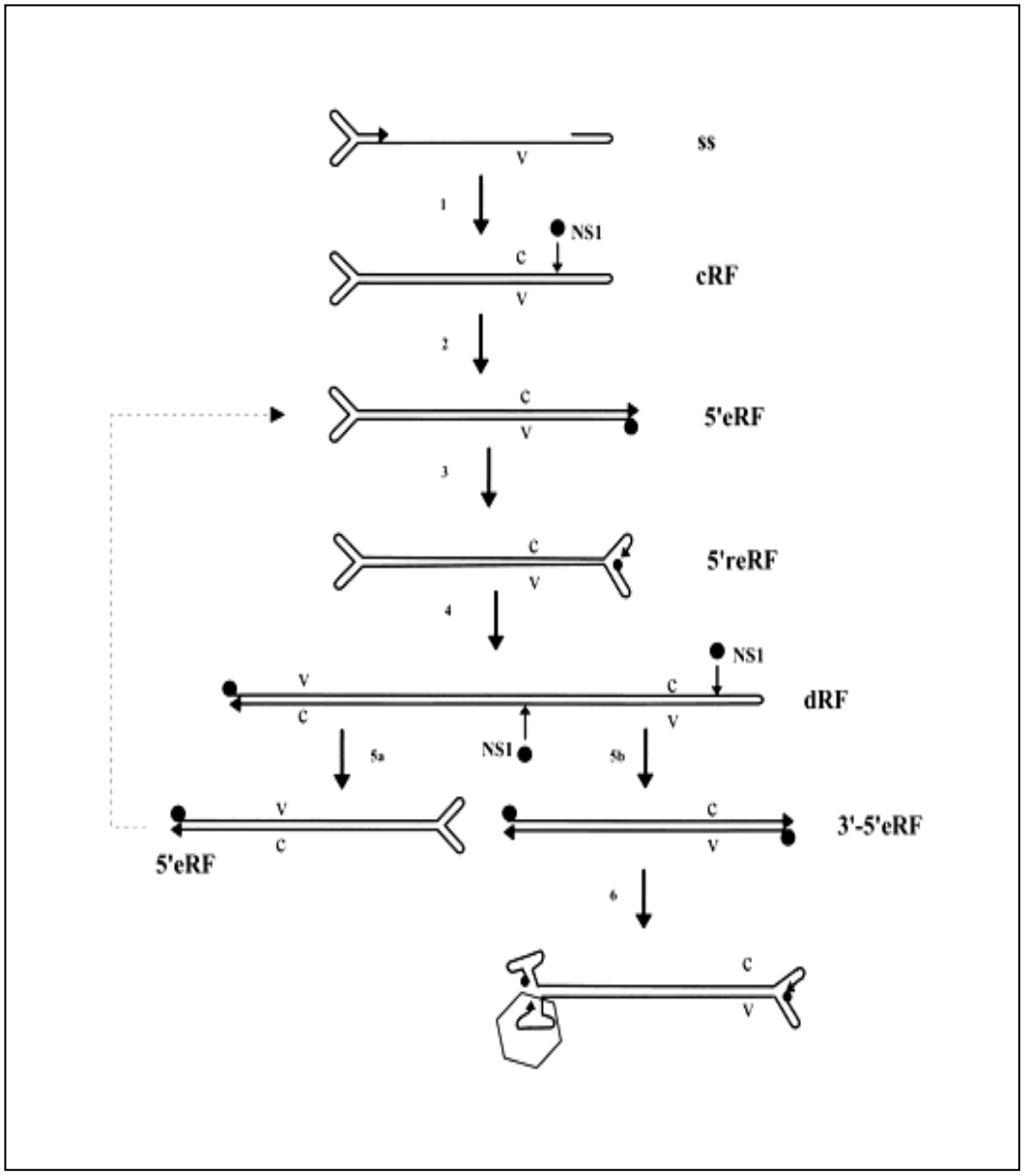


Figure 3. Proposed Modified Rolling Hairpin Model for MVM DNA Replication. Small filled circle represent NS1. Small arrowheads indicate DNA 3' ends. Ss; single stranded DNA, cRF; covalently closed replicative form, eRF; extended replicative form, reRF; rabbit-eared replicative form, dRF; dimeric replicative form v; viral strand, c; complementary strand. Open polygon in step 6 represents the capsid (Reprinted from Kuntz-Simon et al; 1999).

In the dimer bridge arrangement, the mismatched nucleotides located on the inboard strand of the hairpin stem (5' GAA), aligned with the doublet (5' GA) on the outboard strand are now base paired with their complementary sequences (Figure 4). This region constitutes the minimal left origin of replication of MVM (19). Here, NS1 viral protein in cooperative interaction with a cellular protein called parvovirus initiation factor (PIF) is able to introduce a single-stranded nick (CTWWTCA) into the lower strand of the dimer junction to initiate replication (20). The PIF binding site overlaps a consensus binding for the CREB/ATF family of host transcription factors. It recognizes the two tetranucleotide (ACGT) motifs within the ATF consensus, located near the NS1 binding site $(ACCA)_2$ (27). The region containing the PIF binding site, including the ATF motif is highly conserved in the 3' hairpin of other parvoviruses related to MVM, such as H-1 and LuIII.

Resolution of the dimer (dFR) generates two new replicative forms, a turn-around replicative form (5'eRF) that re-enters in the cycle as described in (step 3) and the extended left hand termini (3'-5'eRF) that is associated with NS1 (step 5a, 5b). Successive strand displacements will generate single-stranded viral DNAs, which are packaged into pre-formed empty capsids (step 6).

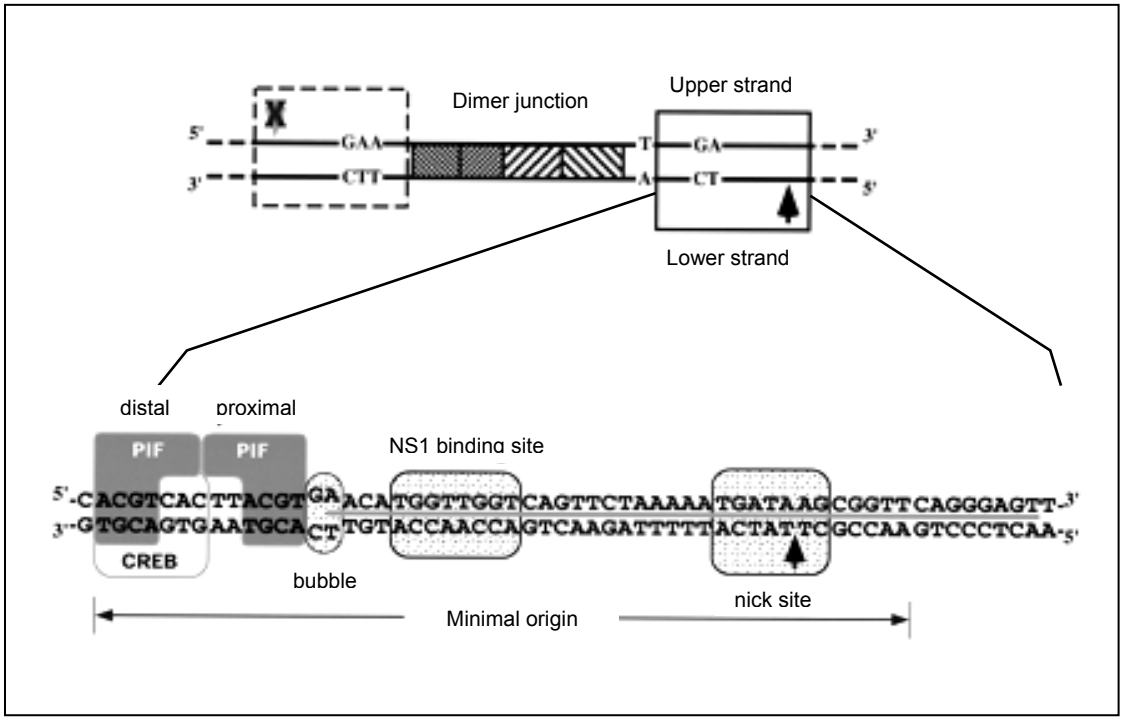


Figure 4. Organization of the MVM dimer bridge. (Top) Organization of the left-end hairpin sequences within the duplex dimer junction generated by replication through the hairpin. The boxed sequence represents the minimum active replication origin and the elements involved in replication. The NS1 box indicates the specific NS1 recognition sequence $(ACCA)_2$. Nick site indicates the position at which NS1 nicks the DNA to initiate replication. The PIF binding site overlaps a consensus binding site for the CREB/ATF family of host transcription factors. The two tetranucleotide motifs bound by PIF are labeled "distal" and "proximal" to indicate their positions relative to the NS1 binding site (Adapted from Christensen et al; 2001).

Christensen et. al (18) propose a molecular model of the parvovirus replication fork (Figure 5). Given it has not yet been to reconstitute replication of any full length parvovirus genomes using only purified cellular components, discrete steps of replication for MVM have been reconstituted in vitro using as templates recombinant NS1, fractionated human cell extracts, and the minimal TC origin cloned in plasmids (28). Nicking and covalent attachment of NS1 to MVM origins liberates the 3' hydroxyl groups necessary for priming DNA replication. Replication from such templates appears to employ a unidirectional leading strand synthesis mechanism. PCNA (proliferating-cell nuclear antigen) and RPA (single stranded DNA replication protein A) have previously been shown to be required for in vitro MVM DNA synthesis (20). The classical components of the leading strand synthesis machinery include PCNA, a ring-shaped homotrimeric protein forming a sliding clamp at the primer template junction, Pol δ, RPA, and the heteropentameric multifunctional preotein RFC, which loads PCNA and Pol δ onto the template. These factors in concert with NS1, promotes processive chain elongation to form the parvoviral replication fork.

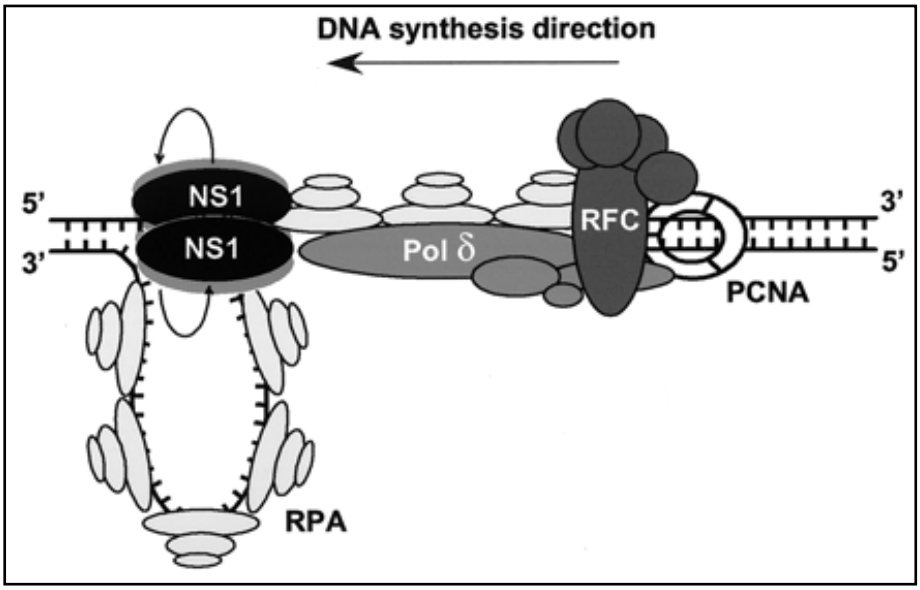


Figure 5. Model of minimal parvovirus replication fork. **NS1** and **RPA** specifically coordinate the progression of the parvoviral replication fork. Subsequent assembly of recombinant leading strand DNA synthesis machinery consisting of replication factor C **(RFC)**, proliferating cellular nuclear antigen **(PCNA)**, and DNA gamma **(Pol δ)** polymerase efficiently catalyzed processive DNA synthesis (Reprinted from Christensen et al; 2002).

Encapsidation Pattern

Parvoviruses encapsidate their single stranded genomes irrespective of its plus or minus polarity, however the ratio varies among members (Table 2). AAV and B19 encapsidate both, plus and minus DNA strands with equal frequency, and have identical termini. Due to the fact that AAV and B19 have identical termini, scientists initially proposed that identical termini were responsible for the encapsidation pattern observed in these viruses. Diffoot et al. (34) demonstrated that LuIII has different termini and encapsidates both strands with equal frequency (6). The autonomous parvoviruses MVM, H-1 encapsidates 90-99% of minus strand and have different termini. LuIII shares over 80% sequence identity with MVM and H-1. Comparison of the MVM and H-1 sequences with the LuIII genome identifies a unique adenine-thymine (A/T)-rich sequence of 47 nucleotides (5'TATGCTCTATGCTTCATATATAT-TATATATATTATTATACTAACTAA3') located at map unit 89, 6 bases downstream from the end of the right open reading frame (ORF).

This A/T rich region is unique to the LuIII genome and has not been found in any of the newly isolated rodent parvoviruses (33). This sequence rich in adenines and thymines interrupts a sequence that, in MVM binds cellular proteins. Therefore, it has been suggested that in LuIII, the A/T-rich region could interfere with the binding of cellular proteins to the viral genome, allowing the virus to encapsidate plus and minus DNA strands with equal frequency (24). Given that this A/T rich stretch is the only sequence in this region of LuIII that differs significantly from that of MVM and H-1, this A/T rich region may play an important role during LuIII replication.

Table 2. Encapsidation pattern of selected parvovirus (9)

VIRUS	TERMINAL HAIRPINS	ENCAPSIDATION PATTERN
B-19 AAV	Identical	50%(+), 50%(-)
MVM H-1	Non identical	99%(-), 1%(+)
LuIII	Non identical	50%(+), 50%(-)

Origins of replication (Autonomously replicating sequence)

Origins of DNA replication are best understood in the yeast *Sacharomyces cerevisiae*. These are sites in which DNA replication is initiated. Generally, replication origins contain internal repeats that are rich in A-T base pairs. Since A-T base pairing is weaker than G-C base-pairing, A-T rich helices make it easier for helicases to open the helix, allowing access of primases and other enzymes to each strand. The replication of yeast chromosomes is accomplished by the activation of multiple *cis*-acting replicators. These replicators were first identified by their ability to promote high-frequency transformation and stable extrachromosomal maintenance of plasmids in yeast (47, 84, 95). These have also been identified in a variety of others species, such as humans and drosophila (44, 97). Given that these replicators confers autonomous replication on episomes and have been extensively characterized they were named autonomously replicating sequence (ARS) elements (23). However, only subsets of ARS elements are active as origins in their native chromosomal context (37).

The structures of the chromosomal replication origin have been extensively studied (58, 75). Each ARS consist of a 100 to 200 bp sequence, with a high A-T content (73 to 82%) (68). ARS have a modular structure and contain an essential match or near match to the 11 bp ARS consensus sequence (ACS) $5'^{T}/_{A}TTTA^{T}/_{C}{}^{A}/_{G}TTT^{T}/_{A}3'$ called element or domain A (11, 98). This domain A is present in most of the ARS sequences. This sequence was extended in ARS 309 of *S. cerevisiae*. The classical 11 bp ACS of *S. cerevisiae* in ARS 309 was a 17 bp AT-rich sequence. Domain A is more diverse than initially thought (5'

WWWWTTTAYRTTTWGTT 3') (90). In addition to domain A, a region 3' to the T-rich strand of the ACS is also required and it is referred to as domain B (16) (Figure 6).

Domain A is recognized and bound by the origin of recognition complex (ORC). ORC is a six-polypeptide complex, essential for the initiation of DNA replication *in vivo* and *in vitro* (8, 71). Its considered to be the putative initiator complex in yeast, because it is bound to the ORI throughout most of the cell cycle (2). Also; its thought to function as a landing pad for others proteins involved in replication (39). The binding of ORC to ARS elements is ATP dependant, and it has recently been shown that ORC is an ATPase whose activity is modulated by sequence-dependent binding to DNA (7, 49). ORC participates in the formation of the pre-replicative complex, which is necessary to establish replication competence (81).

Domain B is composed of several small sequence elements (B1, B2 and B3) that can differ from one ARS to another (75, 92). Despite a sequence homology, some of these elements act as functional analogues. Some mutations in B1 reduce the efficiency of ORC binding, suggesting that B1 functions along with domain A as part of the ORC binding site (75, 91). However, the observation that some B1 mutations reduce ARS efficiency without affecting ORC binding *in vitro* suggest that the B1 element plays an additional role in the replicator activity (75). Domain B2 is a stretch of easily unwound DNA and may affect replication efficiency as a cis-acting regulator (53). The B3 element is a binding site for the ARS binding factor 1 (ABF1) (32). ABF1 is a multifunctional, site-specific DNA binding protein that is essential for cell viability in *Saccharomyces cerevisiae*. It's plays a direct role in transcriptional activation,

stimulation of DNA replication, and gene silencing at the mating-type loci. (62). ABF1 binding sites are found 5' of the T-rich strand of the essential match to the ACS.

In the fission yeast *Schizosaccharomyces pombe*, chromosome fragments capable of autonomous replication are several times larger than the ARS fragments of the budding yeast *S. cerevisiae* (14, 36). Although an 11 bp sequence, similar to that of *S. cerevisiae* can be deleted without any effect on ARS activity (59). Detailed analysis of the three fission yeast ARS elements, have shown that regions containing adenines and thymines asymmetrically on one strand of the DNA duplex are required for ARS activity (23, 36). Although the adenine and thymine rich (A/T-rich) regions are different in size and sequence, and its still not know whether a specific sequence or A/T richness is more important.

Transformation with pNA1, a clone containing exclusively the A/T rich sequence was shown to replicate in yeast (unpublished results) but colonies could not be subculture. To provide any further functions required for replication, possibly present at the right end of the LuIII sequence, a fragment containing map units 88-100 of LuIII were cloned into a yeast vector pGN3 (clone pUra-Lu88-100). Given the similarity of the LuIII A/T rich sequence with the autonomously replicating sequences present at multiples origin of replication in yeast, this putative ARS-like sequence could represent the origin of replication required for the synthesis of the LuIII plus strand DNA.

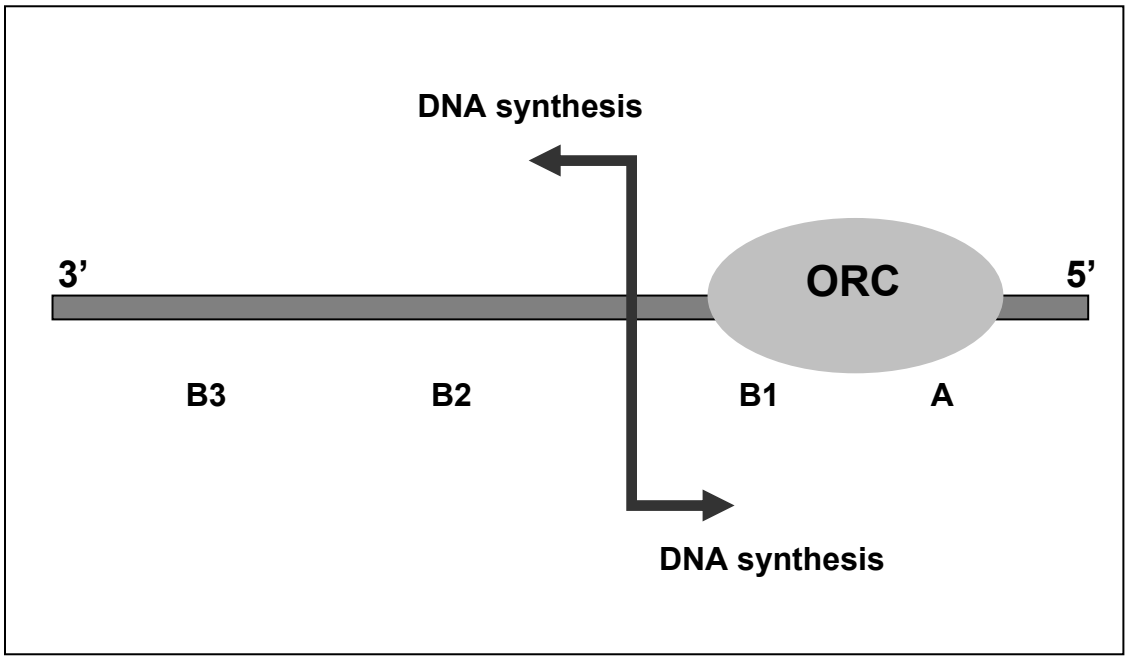


Figure 6. **Modular structure of ARS1 in *Sacharomyces cerevisiae***. ARS 1 In yeast has a modular structure and is composed of A and B domains. A and B1 comprise the ORC binding site, B2 is a DNA-unwinding element, and B3 is the target site of the transcription factor ARS-binding factor 1 (Adapted from Gilbert et al; 2001).

Parvoviruses as vectors for human gene therapy

Gene therapy involves transfer of genetic material to target cells using a delivery system, or vector (85). Members of the parvovirus genus, like dependoviruses and autonomously parvoviruses are currently being studied as vectors for gene therapy.

The advantages of the dependovirus AAV as a gene vector include transduction of both dividing and non-dividing cells (73), mild immune reaction and long-term transgene expression. Their integration mechanisms may be exploited to insert specific genes into several cell types (55, 78, 92). It can integrate into the human chromosome 19 at high frequency and is not associated with any known human disease. The site-specific integration is in the absence of a helper virus and at least mediated by AAV encoded *rep* proteins. Recombinant AAV genomes are constructed by cloning DNA sequences of interest between the AAV inverted terminal repeats ITR's, eliminating the entire coding sequence of the wild type AAV genome. Recombinant AAV vectors do not have *rep* proteins, therefore site specificity of integration is lost. Clinical trials with AAV vectors are underway for treatment of several diseases including cystic fibrosis (42) and hemophilia, whereas success in animal models is encouraging in the development of treatments for high blood pressure, Parkinson's disease, muscular dystrophy, brain tumor and many other conditions. Maeda et al. (57) reported the expression of the β-galactosidase gene in vascular smooth muscle cells, with the AAV-lacZ vector *in vitro*. These finding suggest that AAV-based vectors could be used in cardiovascular gene therapy as well. Also, a novel packaging strategy combining the salient features of the two human

parvoviruses AAV and B19 was developed to achieve erythroid cell-specific delivery as a well expression of the transduced genes. These studies reflect that the AAV-B19 virus hybrid vector system could be used as a tool in the treatment of human diseases affecting cells of erythroid lineage.

In contrast to AAV, autonomous parvovirus can replicate in proliferating human cells without the assistance of a helper virus (60) and do not appear to integrate into cellular chromosomes. Due to the fact that autonomous parvovirus are unable to force resting cells into S-phase and are therefore incapable of undergoing genome replication in quiescent cells, they replicate efficiently in transformed and tumor cell lines of animal and human origin, probably due to the aberrant cell cycle control exhibited by transformed cells. Particularly, H-1 and MVM can suppress the formation of spontaneous and induced tumors in their natural host or others animals. The oncotropic and oncolytic characteristic of parvoviruses appear to be determined by their enhanced DNA replication capacity in transformed human cells, the oncogene and cell cycle dependent activation of the viral early promoter and the oncogene induced cytotoxic activity of the nonstructural viral protein NS1.

MVM, H-1 and LuIII based vectors have been developed in different ways. Vectors may be designed to maintain their oncotropic properties (79). The early promoter and the region encoding the NS proteins have been retained, and genes encoding the structural viral proteins were replaced by various transgenes (38, 61). These recombinant viruses expressed the transgenes from the internal P38 promoter, allowing vector DNA to replicate at the same level as standard viral DNA (48).

For example, Clément et al. (22) cloned and sequenced spontaneously occurring defective particles of MVMp; a variant of MVM, with very small genome to identify the minimal *cis*-acting sequences required for DNA amplification and virus production. Some had lost all capsid-coding sequences but were still capable of replicating in permissive cells when non-structural proteins were provided in *trans*. This has led to the design of parvovirus based vectors in which the viral capsid genes have been replaced with genes of interest. Haag et al. (45) generate a recombinant virus of H-1 (rH-1), which carried transgenes encoding either human interleukin 2 (IL-2) or monocyte chemotactic protein 1 (MCP-1) in replacement of part of the capsid genes. These proteins play an important role in immune response, these genes code for a growth factor for cytotoxic and helper T cells, and a protein involved in the recruitment of monocyte and macrophages, respectively. Their data presented high levels of recombinant cytokines when human tumor cells (HeLa) were infected with parvovirus H-1 derived vectors.

Maxwell et al. (61) demonstrated that a recombinant clone of LuIII could replicate and package in cells transiently co-transfected with a replication defective helper construct. They replaced the viral nonstructural and capsid genes of the LuIII genome with the reporter genes, luciferase and B-galactosidase, respectively. They suggest that the terminal sequences were deleted from the helper virus to minimize recombination between the helper and transducing genomes.

These investigations in combination with the oncospecificity, high transduction efficiencies and lack of pathogenicity present in rodent parvovirus probe that MVM, H-1 and LuIII are promising tools for tumor-cell targeted gene therapy (40, 60, 80).

CHAPTER III
Materials and Methods

Construction of pUra-Lu88-100 clone

pGLu883, a construct that consists of the full length genomic clone of parvovirus LuIII was fully digested with the restriction enzymes *Bam* HI (G↓GATCC) and *Xba* I (T↓CTAGA) (Figure 7) (Roche Molecular Biochemical's, Indianapolis, IN). The LuIII fragment containing map units 88-100 (nucleotide 4527 to 5135) was isolated and purified with Gene Clean Spin Kit® (Q.BIOgene, Carlsbad, CA). pGN3, a plasmid containing the auxotrophic marker URA3, isolated from *Saccharomyces cerevisiae*, cloned into the *Hind* III (A↓AGCTT) site of pUC19, was digested with the restriction enzymes *Bam* HI and *Xba* I. The 608 bp fragment of LuIII was ligated into the pGN3 *Bam* HI and *Xba* I sites using T_4 DNA ligase (Rapid DNA Ligation Kit, Roche Molecular Biochemical's Indianapolis, IN) at room temperature for one hour. This clone was called pUra-Lu88-100 (Figure 8).

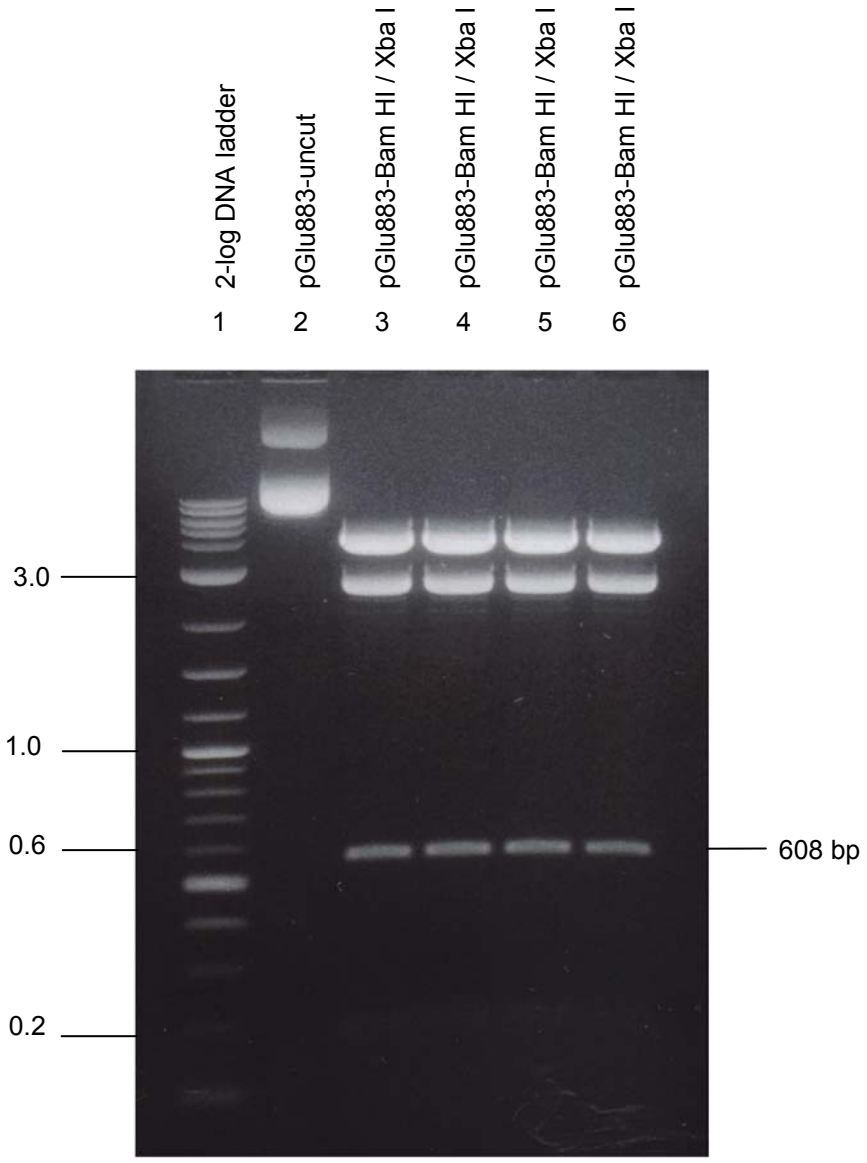


Figure 7. Digestions of the genomic clone of LuIII, pGlu883 with *Bam* HI and *Xba* I. Lane 1: 2 log ladder (New England Biolabs), lane 2: pGLu883 uncut, lanes 3-6: *Bam* HI and *Xba* I digestions. Samples were electrophoresed on a 1% agarose-TBE gel at 70 V.

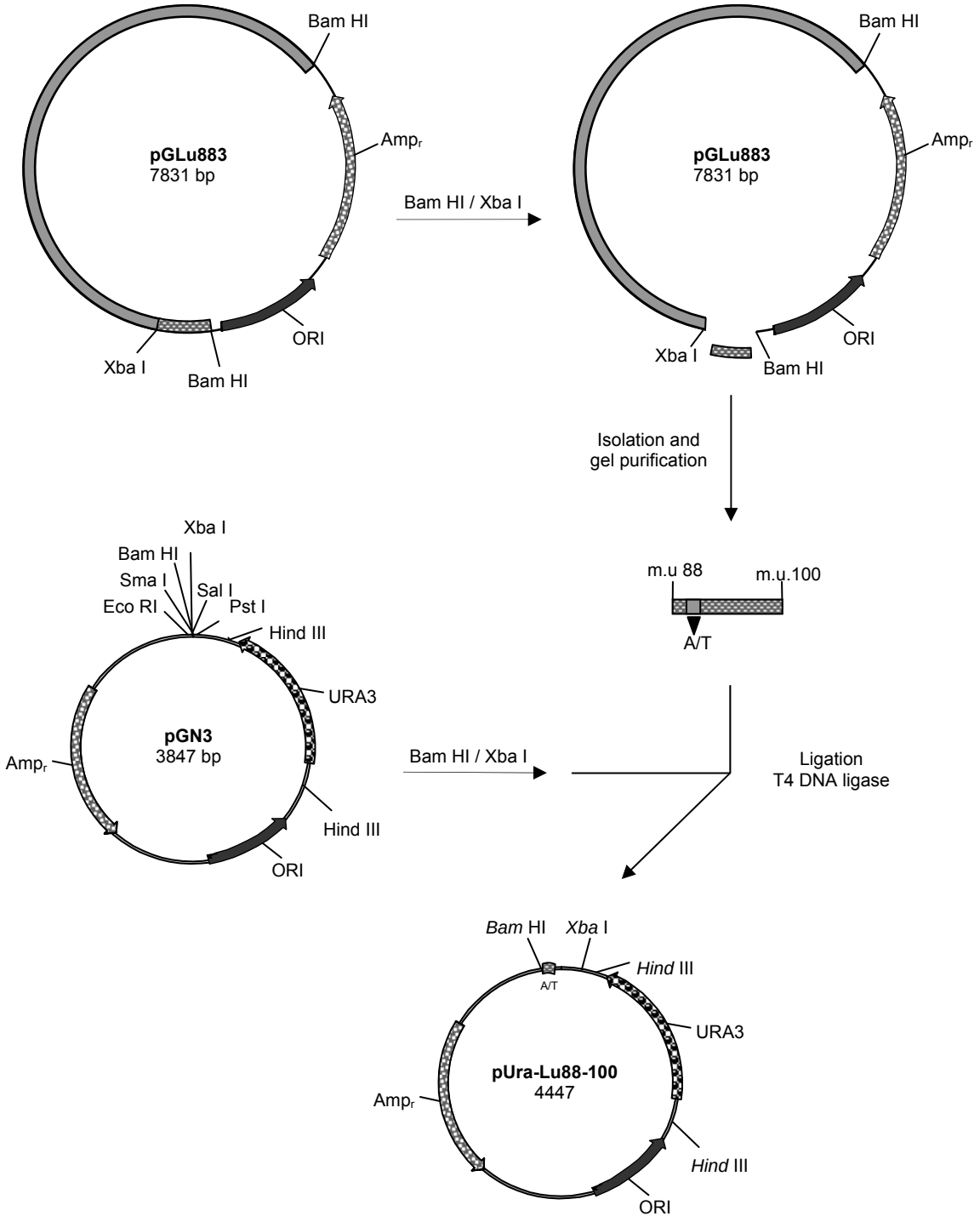


Figure 8. Strategy used to construct pUra-Lu88-100. Restriction sites of the multiple cloning site (MCS) in pUC19, the uracil (URA3) and the ampicillin (Amp$_r$) genes are shown. Map units (mu) 88-100 of LuIII are cloned into the *Bam* HI and *Xba* I sites of pGN3.

Preparation and Transformation of Bacterial Competent Cells by the Calcium Chloride Method

Competent cells were prepared inoculating a single colony of *E. coli* DH5α (ϕ80dlacZΔM15, recA1, gyrA96, thi-1, hsdR17 (r_k^-, m_k^+), supE44, relA1, deoR, Δ(lacZYA-argF)U169) into two ml of LB (1% Bacto® tryptone, 0.5 % Bacto® yeast extract, 86 mM NaCl and 1 M NaOH) broth. Cells were grown overnight at 37 °C with constant shaking. One milliliter of the overnight culture was inoculated into 100 ml of LB and incubated with constant shaking at 37 °C for three to four hours. Cells were harvested by centrifugation at 2,500 rpm for 10 minutes at 4 °C. The pellet was resuspended in 25 ml of 50 mM $CaCl_2$ and placed in ice for 30 minutes. Cells were then centrifuged at 2,500 rpm for 10 minutes and resuspended in 5 ml of 50 mM $CaCl_2$/20% glycerol. Aliquots of 100 µl of competent cells were made. Approximately 25 nanograms of the ligation mixture were added to the cells. Twenty nanograms of pUC19 were added to a 100 µl aliquot of cells to be used as a standard control, and the cells with no DNA were used as a negative control. The tubes were placed on ice for 30 minutes. The samples were then heat-shocked for 2 minutes at 42 °C and immediately inoculated into 100 µl LB medium without ampicillin. Cells were incubated at 37 °C with constant shaking for one hour. Subsequently, cells were plated in LB medium containing ampicillin (50 mg/ml) and 50 µl of X-gal, and then incubated overnight at 37 °C.

Isolation and Characterization of pUra-Lu88-100

The resultant transformants were inoculated in 2.5 ml of LB broth with ampicillin, and incubated overnight at 37 °C with constant shaking. Extraction and

purification of plasmid DNA from the cultures was done using the Small Scale Plasmid Purification Protocol (5). Independent samples of the extracted DNA were digested with restriction enzymes *Bam* HI, *Xba* I and *Hind* III independently and loaded into a 1 % agarose-TBE gel for separation by electrophoresis.

Southern Hybridization

DNA was transferred passively on to a Zeta-Probe® GT membrane (Bio-Rad, Hercules, CA) using 0.4M NaOH. After transfer the membrane was washed briefly in 2X SSC, and then sealed in a plastic bag with 20 ml of pre-hybridization solution (Roche Diagnostics, Indianapolis, IN) and incubated at 37 °C for 2 hours with constant shaking. The A/T probe was made with the Dig Oligonucleotide Tailing Kit (Boehringer Mannheim GmbH, Bedford, MA). The pre-hybridization solution was replaced with 10 ml of fresh hybridization solution containing denatured digoxigenin labeled A/T probe and then incubated overnight at 37 °C with a slow constant shaking. Washes were performed twice at 42 °C with 50 ml of 2X SSC / 0.1% SDS for 20 minutes and once in 0.1X SSC / 0.1%SDS for 1 hour at 42 °C. Detection of the Dig-labeled DNA was done following the Chemiluminescent Detection Protocol provided by the DIG System User's Guide for Filter Hybridization (Roche Diagnostics, Indianapolis, IN).

A second hybridization was performed using the URA3 gene as probe. The URA3 probe was synthesized with the Random Primed DNA Labeling Kit (Boehringer Mannheim GmbH, Bedford, MA). The membrane was incubated with 20 ml of pre-hybridization solution in a plastic bag at 44 °C for 2 hours. The pre-hybridization solution was replaced with the URA3 probe and incubated overnight at 44 °C. The membrane was washed twice at 65 °C with 50 ml of 2X SSC / 0.1% SDS for 20

minutes and once in 0.1X SSC / 0.1%SDS for 1 hour at 65 °C. The detection was performed using the method described above.

DNA Sequencing

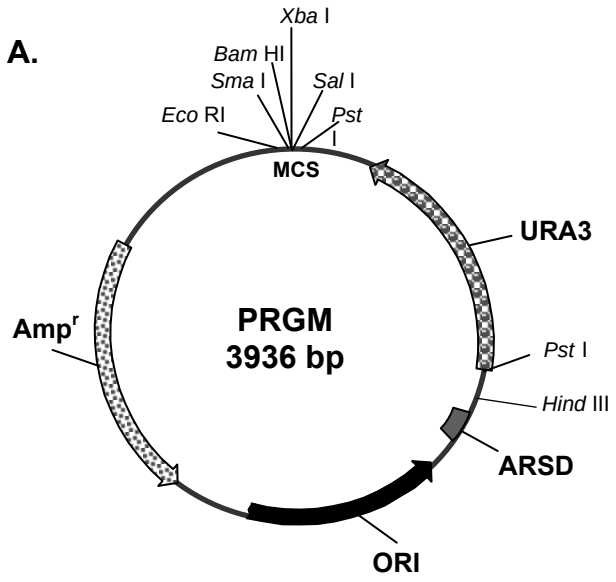
The insertion of LuIII sequences into pGN3 was confirmed by the dideoxy sequencing method using the T7 Sequenase Version 2.0 DNA Sequencing Kit ((United States Biochemicals, Cleveland, OH). Sequencing of plasmid DNA isolated from yeast and electroporated into *E.coli* DH5α cells was done at the New Jersey Medical School, Molecular Resource Facility, (Newark, NJ 07103). The primers used for forward and reverse sequencing were 10773 and M13 respectively.

Preparation of Yeast Electrocompetent Cells

Yeast electrocompetent cells were prepared by inoculating 2.5 ml of YPD broth with a freshly isolated single colony of *Saccharomyces cerevisiae* (SEY 2108) (MATα. ura 3^{-}52 leu 2-3, -112 suc2-Δ9Δprc1:Leu2^{+2}) and incubated at 30 ˚C overnight. The 2.5 ml culture was inoculated into 250 ml of YPD and grown overnight at 30 ˚C. The cells were grown to an OD_{600} of 1.3 to 1.5 and harvested by centrifugation at 4,000 rpm for 5 minutes at 4 °C. The pellet was resuspended in 170 ml of ice-cold sterile water and centrifuged as indicated above twice. After the second wash the cells were resuspended in 10 ml of ice-cold sterile, 1 M sorbitol and centrifuged at 4,000 rpm for 5 minutes at 4 °C. The pellet was resuspended in 0.17 ml of 1M sorbitol and aliquots of 65 µl were prepared.

Experimental controls for yeast transformation

pRGM, a plasmid that contains the auxotrophic marker URA3 of *S. cerevisiae* and an ARS sequence isolated from *Debaryomyces hansenii* was used as a positive control (65). pGN3, a plasmid that has the auxotrophic marker URA3 but lacks the ARS sequences and is not capable of autonomous replication in yeast was used as negative control (Figure 9).



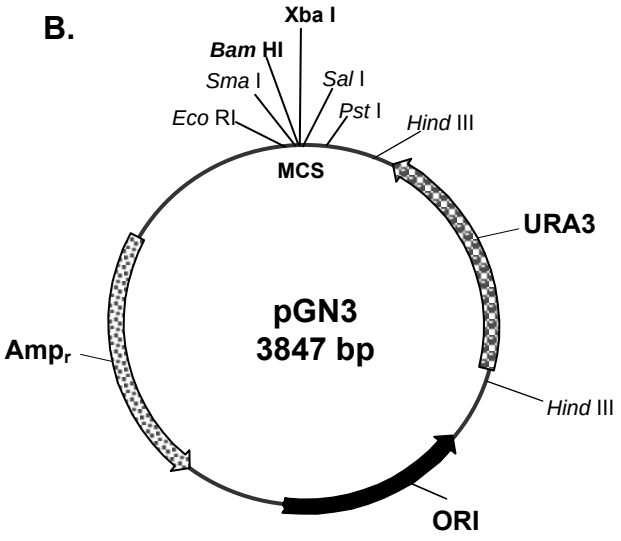


Figure 9. Genomic Maps of experimental control plasmids for yeast transformation: A. pRGM, positive control and **B.** pGN3, negative control. **MCS**: multiple cloning site, **URA 3**: auxotrofic marker, **ARSD**: an autonomously replicating sequence from *D. hansenii*, **ORI**: origin of replication, **Amp^r**: bacterial gene for resistance to ampicillin.

Transformation of Yeast Electrocompetent Cells

Five independent transformations of *S. cerevisiae* were performed. Concentrations of plasmid DNA used are shown in Table 4. Plasmid DNA's were added to the electrocompetent cells and incubated on ice for 5 minutes. The mixture was then transferred to an electroporating 2mm cuvette. The electroporator Bio-Rad Gene Pulser® (Bio-Rad Laboratories, Hercules, CA) was set at 1.10 Kv, 25 µF and 200 Ohms. After the pulse, the cuvette was removed from the electroporator and one ml of ice-cold, 1M sorbitol was added. Amounts of 150 and 500 µl of the electroporated mixture were plated on SD ura- plates and incubated at 30 °C for five days.

Extraction and Purification of Total Yeast DNA

Transformants were inoculated in SD ura- broth and incubated overnight at 30 °C with constant shaking. The isolation of plasmid DNA from the culture was performed using the Y-Der™ Yeast DNA Extraction Kit (Pierce, Rockford, IL). One µl of the isolated total DNA was used to transform *E.coli* DH5α electrocompetent cells. The Small Scale Plasmid Purification Protocol was used to extract and purify the transformants recovered from the culture. The samples were digested with *Bam* HI and *Xba* I, and electrophoresed on a 1% agarose gel in 1X TBE and transferred to a Zeta Probe membrane for Southern Blot Analysis. Hybridization with a digoxigenin labeled A/T and ura 3 probe was done as previously described using the DIG System User's Guide for Filter Hybridization.

Preparation and Transformation of *E. coli* DH5α electrocompetent cells with DNA isolated from yeast transformants

A single colony of *E. coli* DH5α was inoculated into two ml of LB broth. Cells were grown overnight with constant shaking at 37 °C. One ml of the overnight culture was inoculated into 100 ml of LB at 37 °C and was grown to an $O.D._{600}$ of 0.5 to 0.6. Cells were chilled in ice water for 15 minutes, and then harvested by centrifugation at 5,000 rpm for 15 minutes at 4 °C. The pellet was resuspended in 5 ml of cold water and then washed two times in cold water followed by centrifugation at 5,000 rpm for 20 minutes. The supernatant was removed and the volume of the pellet was calculated and an equal amount of water was added. Aliquots of 100 µl of cells were prepared.

One microliter of the total DNA isolated from yeast were added to a 100 µl of *E.coli* DH5α electrocompetent cells in a 2mm cuvette. The electroporator was set at 2.5 Kv, 25 µF and 400 Ohms. After the pulse, cells were inoculated into 1 ml of SOC medium without ampicillin and incubated at 37 °C with constant shaking for one hour. Amounts of 50 and 150 µl of cells were plated on LB with ampicillin (50 mg/ml) and 50 µl of X-gal and incubated overnight at 37 °C.

CHAPTER IV
Results and Discussion

Sequence analysis of the LuIII genome revealed the presence of an A/T rich region unique in LuIII, which shares sequence homology with the autonomously replicating sequences (ARS) characterized in yeast. To test the possible role of the A/T rich region as an origin of replication in yeast, a clone containing only the LuIII A/T rich region was constructed (pNA1) and subsequently transformed into *Saccharomyces cerevisiae* (SEY-2108,ura 3-)(3). It was observed that yeast transformants containing the pNA1 plasmid grew slowly and could not be sub-cultured. The explanation given to this observation was that the plasmid pNA1 did not contain all the elements necessary to function as an ARS. The purpose of this work was to determine if the LuIII A/T rich sequence requires additional sequences present across the right terminus to enhance the ARS activity observed by Arroyo (3). To study this possibility, a clone (pUra-Lu88-100) containing LuIII sequences from the A/T rich sequence to the end of the right terminus of the viral genome (nucleotides 4527 to 5135) was constructed.

Transformation of *E.coli* DH5α cells with the possible pUra-Lu88-100 recombinants resulted in a total of 120 colonies (Table 3). No growth was expected for the restriction enzyme control (pGN3 digested with *Bam* HI and *Xba* I), yet a total of 8 colonies were obtained; these likely represent supercoiled molecules that were not digested. The transformation efficiency was 1.3×10^{7} CFU/μg DNA.

Table 3. Transformants of *E.coli* DH5α

SAMPLES	VOLUME SEEDED/ TOTAL VOLUME	CFU**
Cells without DNA	50 μL/200μL	0
	100 μL/200μL	0
pUC 19 (standard control) [10 ng]	50 μL/200μL	4,600
	100 μL/200μL	8,500
pGN3 digested with Bam HI and Xba I (restriction enzyme control) [40 ng]	50 μL/200μL	3
	100 μL/200μL	5
Ligation of pGN3 + LuIII nt. 4527 to 5135 [25 ng]*	50 μL/200μL	45
	100 μL/200μL	75

*Concentration is based on the concentration of pGN3 in the ligation mixture
**Colony forming units

To check for the desired insert in the recombinant molecules plasmid DNA isolated from sixteen bacterial transformants was digested with restriction enzymes *Bam* HI, *Xba* I and *Hind* III, and the fragments were resolved by electrophoresis (Figure 10A). Lane 1 contains the 1Kb ladder marker, lane 2 contains pGlu883 digested with *Bam* HI and lane 3 contains pGN3 digested with *Hind* III. Lanes 4 and 5 contain the possible recombinant clones pUra-Lu88-100 digested with *Bam* HI and *Xba* I. If the construction is correct this digestion should generates two fragments of ~3,841 and ~608 bp. As expected, the first fragment of ~3,841 bp corresponds to the pGN3 vector DNA, and the second fragment of ~608 bp corresponds to the LuIII nucleotides (4527-5135). Lanes 6 and 7 contains the same recombinant clone pUra-Lu88-100 digested with *Hind* III. Once again as expected, two fragments of ~3,288 and ~1,161 bp were generated, corresponding to the pUC19 vector DNA containing the LuIII sequences and the URA 3 gene respectively. Southern Blot Analysis of the DNA samples described above was performed using a digoxigenin labeled LuIII AT-rich specific probe (Figure 10B). The signals obtained identify the fragments that contain the LuIII A/T rich sequence. The restriction analysis performed resulted in the expected fragments and the Southern Blot signals confirmed the insertion of map units 88-100 of LuIII into the pGN3 vector DNA.

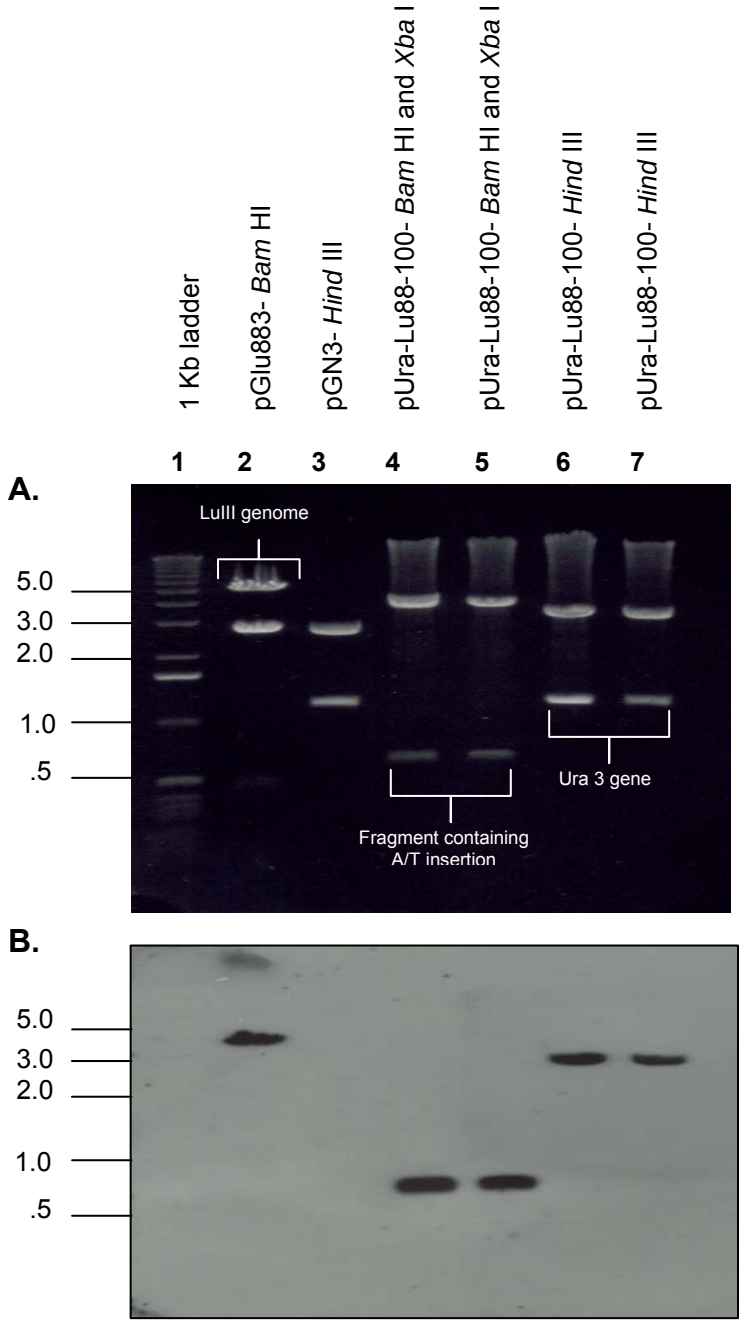


Figure 10 A. Restriction analysis of possible recombinant clones pUra-Lu88-100 isolated from *E.coli* DH5α. Samples were electrophoresed on a 1% agarose-TBE gel for three hours at 80V. **B**. Southern Blot Analysis of pUra-Lu88-100 isolated from E.coli DH5α. The probe used was single strand LuIII A/T rich sequence labeled with Digoxigenin-11-dUTP.

Once the clone was characterized, pUra-Lu88-100 was used to transform *S. cerevisiae* (SEY2108,ura3-). Independent transformations into *S. cerevisiae* were performed, using different concentrations of DNA samples as described below (Table 4). Positive control, pRGM, resulted in round, white and smooth colonies. The negative control, pGN3, should not replicate in yeast because it lacks an ARS element, nevertheless three to five colonies were obtained. These colonies may be the result of integration of pGN3 into the yeast chromosome by homologous recombination (13); therefore the Ura 3 gene could be expressed during chromosome replication, resulting in a low efficiency of transformation in selective media.

Colonies transformed with pUra-Lu88-100 grew normal in size, color and were smooth like the positive control, pRGM, yet these colonies were sub-cultured *Saccharomyces cerevisiae* (SEY-2108,ura 3-) until the third passage. This loss could be due to the stability of the ARS-like plasmid in these cells. The ARS plasmids may also have a centromere or CEN element, which mediates the attachment of the plasmid to the mitotic spindle, ensuring segregation to both mother and daughter cells during replication (63). pUra-Lu88-100 does not contain the CEN element, therewith after a number of generations the loss of the plasmid providing the Ura gene is possible.

Table 4. Transformation assays in *Saccharomyces cerevisiae*, Ura3- with DNA samples.

Sample		DNA conc. ng/µL	Colonies	Col/µg
Positive control	pRGM	3.5	45	12,857
		3.5	50	14,285
		35	22	628
		87.5	222	2,537
		87.5	9	102
Negative control	pGN3	3.5	3	857
		3.5	3	857
		3.5	3	857
		35	5	102
		122.5	3	24
Recombinant clone	pUra-Lu88-100	3.5	31	8,857
		3.5	19	5,428
		35	36	1,028
		122.5	26	212
		122.5	35	285

One microliter of total DNA samples purified from yeast were used to transform *E.coli* DH5α (Table 5). The transformation efficiency was 1.7 x 10^6 CFU/μg DNA. To determine if the original recombinant clone pUra-Lu88-100 was recovered from *S. cerevisiae* transformants, samples were subjected to restriction enzyme analysis; samples were digested with *Bam* HI and *Xba* I (Figure 11). Lane 1 contains the 2-log DNA ladder marker and lane 2 contains pGlu883 digested with *Bam* HI and *Xba* I. Lanes 3 to 6 contain samples isolated from *E. coli* digested with *Bam* HI and *Xba* I. As expected, two fragments of ~3841 and ~608 bp were generated corresponding to the pGN3 vector DNA and the LuIII nts. 4527-5135 respectively.

The possible pUra-Lu88-100 were also digested with *Hind* III (Figure 12 A). Lane 1 contains the 1Kb ladder marker, lane 2 contains pGlu883 digested with *Bam* HI and *Xba* I and lane 3 contains pGN3 digested with *Hind* III. Lanes 4 to 7 contain the possible pUra-Lu88-100 digested with *Hind* III. As expected two fragments of ~3,288 (correspond to pUC19 and LuIII sequences) and ~1161 (correspond to URA gene) were generated. Lane 8 contains an uncut sample of the original clone pUra-Lu88-100. Southern Blot Analysis of these samples using the digoxigenin labeled A/T probe (Figure 12 B) confirm that the LuIII A/T rich sequence is present in these plasmids.

Table 5. *E. coli* DH5α transformants obtained from total DNA preparations from *S. cerevisiae*

SAMPLES	CFU**
PUra-Lu88-100*	3
	0
	1
	5
pRGM*	2
	5
	2
	0
pUC 19 [10 ng]	4,250
Cells without DNA	0

*DNA samples purified from yeast transformants
**Colony forming units

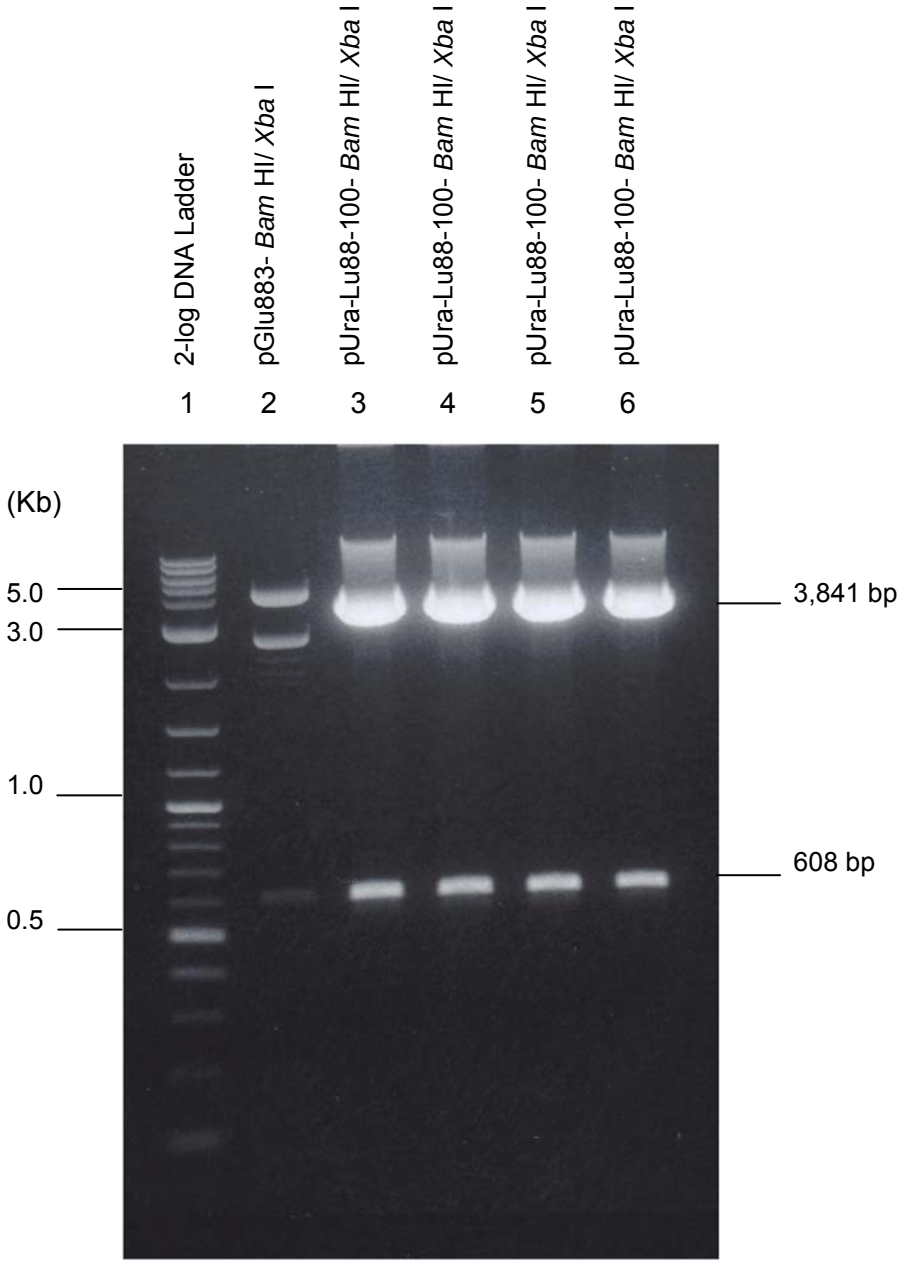


Figure 11. Restriction Analysis of pUra-Lu88-100 with *Bam* HI and *Xba* I. Lanes 3-6 are samples of pUra-Lu88-100 isolated from *E.coli* after transformation into *S. cerevisiae.* These were digested with *Bam* HI and *Xba* I and electrophoresed on a 1.2% TBE agarose gel for four hours at 70V.

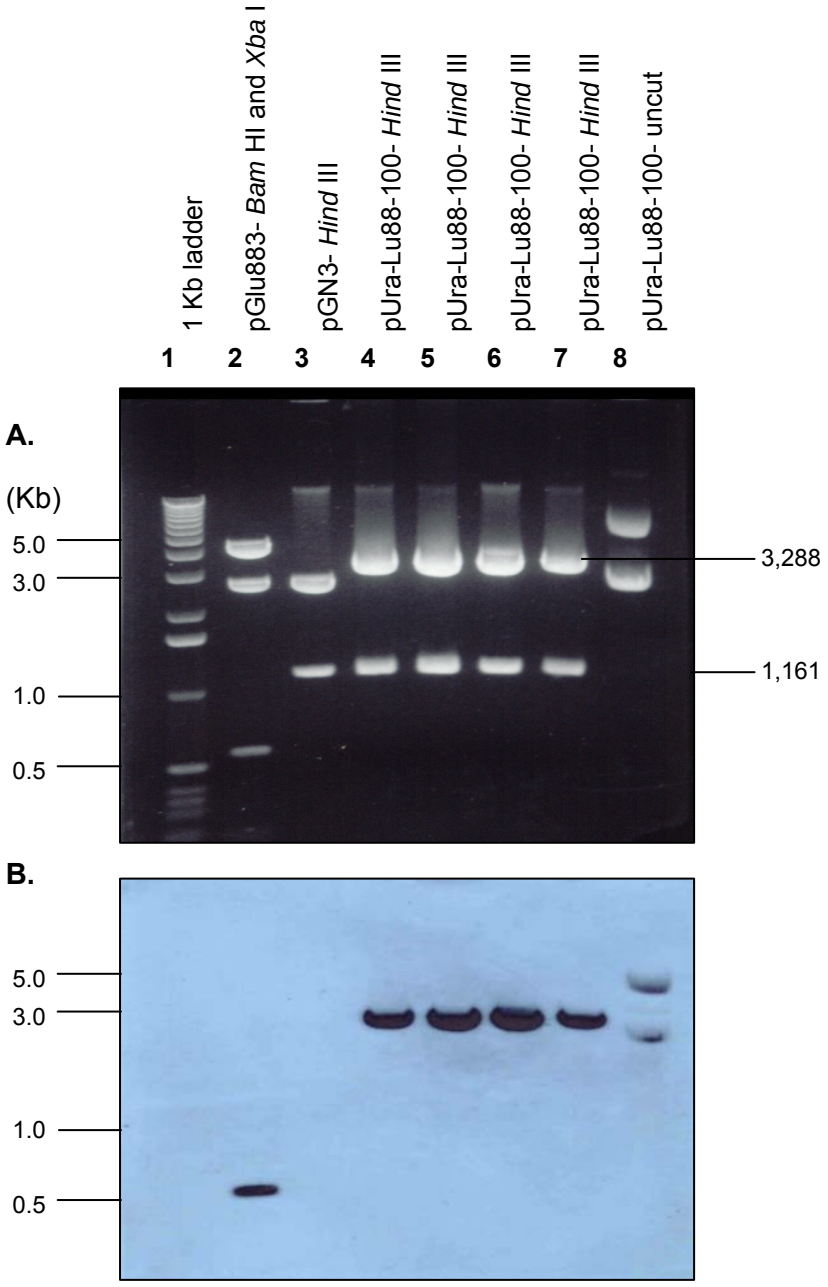


Figure 12 A. **Restriction Analysis of pUra-Lu88-100 recovered from yeast with Hind III**. Lanes 4-7 are samples of DNA isolated from *E.coli* after transformation into *S. cerevisiae* digested with *Hind* III. Samples were electrophoresed on a 1% agarose-TBE gel at 70V. **B**. Southern Blot Analysis of pUra-Lu88-100 isolated from *E.coli* after transformation in *S. cerevisiae*. The probe used was the LuIII A/T fragment labeled with Digoxigenin-11-dUTP.

A second analysis using the URA 3 probe, on other DNA samples recovered from *E. coli* digested with *Hind* III was performed (Figure 13 A). Lane 1 contains the 1Kb ladder marker, lane 2 contains pGlu883 digested with *Bam* HI and *Xba* I and lane 3 contains pGN3 digested with *Hind* III. Lanes 4 to 7 contain the possible pUra-Lu88-100 digested with *Hind* III. Two fragments of ~3,288 and ~1161 were generated. Lane 8 contains an uncut sample of pRGM isolated from *E.coli*. Southern Blot Analysis was performed and the probe used was the random primed labeled URA 3 fragment (Figure 13 B). The positive signals obtained do correspond to the fragments where the URA 3 gene is present. The restriction analysis resulted in the expected fragments and the signals observed on the Southern confirmed the presence of the LuIII insertion and the URA 3 gene. In addition to this techniques, a sequencing analysis of plasmid DNA isolated from yeast and electroporated into *E.coli* DH5α cells was done at the New Jersey Medical School (Figure 14). The primers used for forward and reverse sequencing were 10773 and M13 respectively. This data confirms that the original pUra-Lu88-100 was recovered from yeast transformants.

The results presented here suggest that the recombinant clone pUra-Lu88-100 exists in a free state in yeast, and that it contains the elements necessary to maintain a limited autonomous-like replication in yeast. In order to identify which sequences across the insertion could be responsible for the pUra-Lu88-100 replication, sequence comparison between the LuIII viral genome and the ARS elements were performed (Figure 14). This analysis revealed the presence of some imperfect copies of the known ARS consensus sequences at the right terminus of the viral genome. As observed the LuIII right terminus exhibits regions that are rich in adenines and thymines downstream the A/T rich region. Detailed analysis of fission yeast ARS

elements have shown that regions containing adenines or thymines asymmetrically on one strand of the DNA duplex are required for ARS activity.

The ARS sequences found in yeast act as origins of replication in the yeast chromosomes (58), therewith the ARS-like function observed in pUra-Lu88-100 suggests the presence of an origin of replication at the right terminus of LuIII genome. This origin at map unit 88-100, if present could be responsible for the synthesis of plus strand by LuIII and as a result the unique encapsidation pattern observed for LuIII.

The rolling circle replication model (RCR) proposed for the replication of the autonomous parvoviruses can explain the replication for viruses that encapsidate mainly minus strand such as MVM and H-1. This is not the case for LuIII, which encapsidates both strands of DNA with equal frequency. Initially, it was thought that the presence of identical termini was responsible for the encapsidation pattern observed by LuIII, based on B19 and AAV that have identical termini and encapsidate their strands with equal frequency. However, LuIII has non-identical termini like MVM and H-1. Comparing the LuIII genome with MVM and HI revealed the presence of a unique A/T rich region of 47 nucleotides (5'TATGCTCTATGCTTCATATAT-ATTATATATATTATTATACTAACTAA3') located at the right end at map unit 89 of LuIII termini. Taking into account the results described above, it is possible that the A/T rich region with the adenines or thymines rich regions present at map units 88-100 provide an intrinsic thermodynamic instability between the double helices. This event could result in DNA unwinding so that cellular proteins could binds to initiate replication as has been described for eukaryotic chromosomal replication origins.

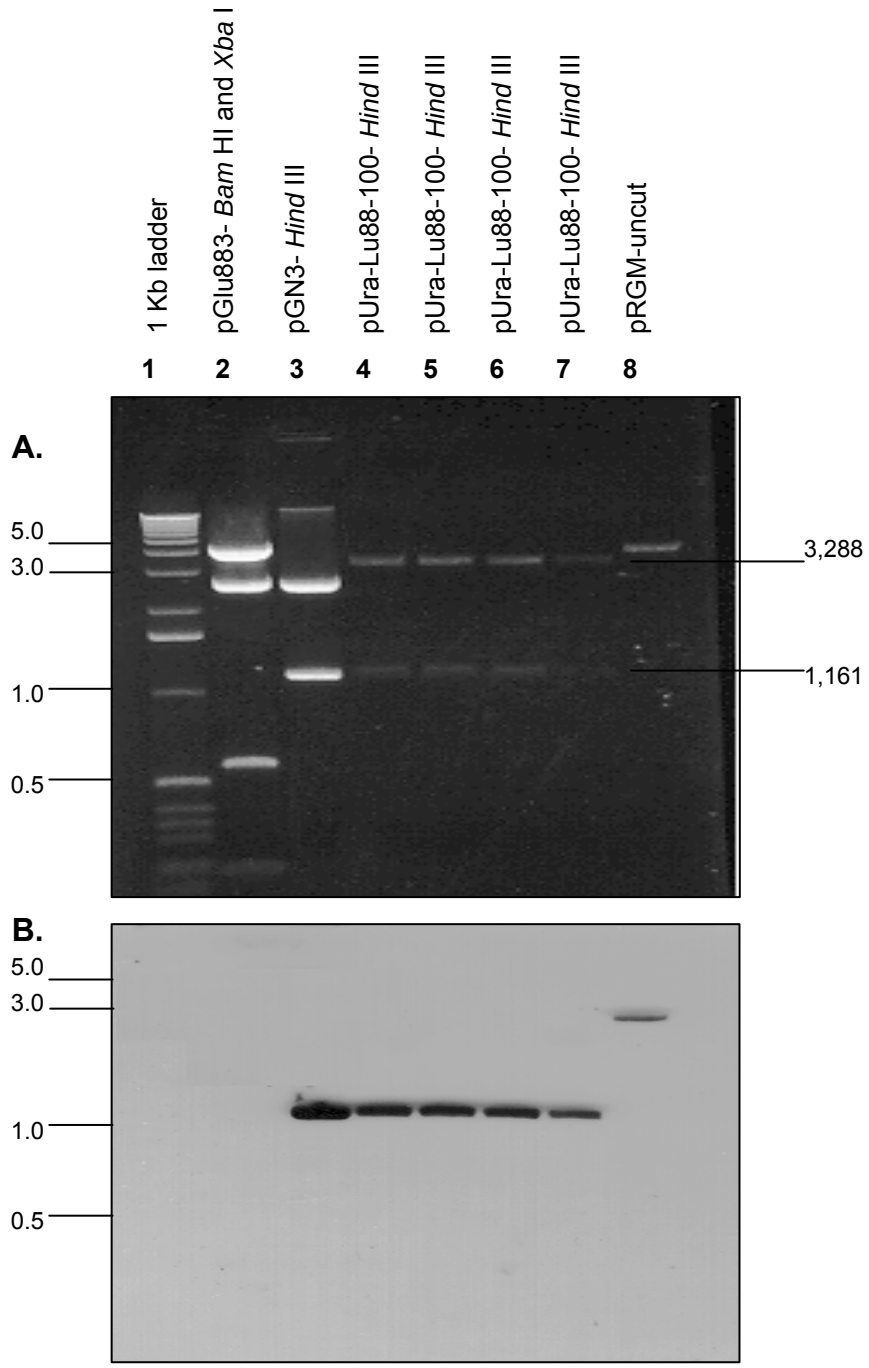


Figure 13 A. Restriction Analysis of pUra-Lu88-100 isolated from *E.coli* after transformation into *S. cerevisiae* with *Hind* III. Samples were electrophoresed on a 1% TBE agarose gel for three hours at 80V. **B**. Southern Blot Analysis of pUra-Lu88-100 isolated from *E.coli* after transformation in *S. cerevisiae*. The probe used was the Ura3 fragment random primed labeled with Digoxigenin-11-dUTP.

A.pUra-Lu88-100 Primer(M13R) URA3 gene

```
      Hind III
CGCCAAGCTT TTTCTTTCCA ATTTTTTTTT TTTCGTCATT ATAAAAATCA
TTACGACCGA GATTCCCGGG TAATAACTGA TATAATTAAA TTGAAGCTCT
AATTTGTGAG TTTAGTATAC ATGCATTTAC TTATAATACA GTTTTTTAGT
TTTGCTGGCC GCATCTTCTC AAATATGCTT CCCAGCCTGC TTTTCTGTAC
GTTCACCCTC TACCTTAGCA TCCCTTCCCT TTGCAAATAG TCCTCTTCCA
ACAATAATAA TGTCAGATCC TGTAGAGACC ACATCATCCA CGGTTCTATA
CTGTTGACCC AATGCGTCTC CCTTGTCATC TAAACCCACA CCGGGTGTCA
TAATCAACCA ATCGTAACCT TCATCTCTTC CACCCATGTC TCTTTGAGCA
ATAAAGCCAT ATAACAAAAT CTTTGTCGCT CTTCGCAATG TCAACAGTAC
CCTTAGTATA TTCTCCAGTA GATAGGGAGC CCTTGCATGA CAATCTGCTA
ACATCAAAAG GCCTCTAGGG TCCTTTGG
```

B.pUra-Lu88-100 primer (10773F)

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                    URA 3                          Hind III  Sph I
GAAATCAAAA AAAAGAATAA AAAAAAAATG ATGAATTGAA AAGCTTGCAT
     Pst I    Sal I     Xba I
GCCTGCAGGT CGACTCTAGA CCTGTTGCTA GAAACACTTA CTAACTAACT
ATGCTCTATG CTTCATATAT ATTATATATA TTATTATACT AACTAACCAT
GTTTACTCTT ACATTACTTC ATATAATATT AAGACTAATA AAAATACAAC
ATAGAAATAT AATATTACAT ATAGATATAA AGAATAGAAT AATATGGTAC
TTACTTACTG TTAGAAATAA TAGAACTTTT GGAATAACAA GATAGTTAGT
TGGTTTATGT TATATAGAAT ATAAGAAGAT GATGTACAAA GAATAAAAGG
GTGGGAGGGT GGTTGGTTGG TACTCCCTTA GACTGAATGT TAGGGACCAA
AAAAATAATA AAATTCTTGA AAACCCAACA AGGACTACTG TCTATATTCA
GTGAACCAAC TGAACCATTA GTATCAATAT GTTTTTAG
```

Figure 14. **Sequence analysis of pUra-Lu88-100 recovered from yeast**. A. Ura3 gene nucleotide(2627 to 523). B. The underlined sequence represents a Ura3 segment and the bold sequence corresponds to parvovirus LuIII insertion. Restriction enzymes are indicated.

```
4451 GGAACCAATGACTACATGAGCATTGAAAGATGGTTACCAACTGCTACTGG
     CCTTGGTTACTGATGTACTCGTAACTTTCTACCAATGGTTGACGATGACC

4501 CAACATAACAAATGTGCCTCTGCTTTCTAGACCTGTTGCTAGAAACACTT
     GTTGTATTGTTTACACGGAGACGAAAGATCTGGACAACGATCTTTGTGAA
                                          ARS 309(82%)
4551 ACTAACTAACTATGCTCTATGCTTCATATATATTTATATATATTATTATAC
     TGATTGATTGATACGAGATACGAAGTATATATAATATATATAATAATATG
                                 LuIII A/T-rich sequence

4601 TAACTAACCATGTTTACTCTTACATTACTTCATATAATATTAAGACTAAT
     ATTGATTGGTACAAATGAGAATGTAATGAAGTATATTATAATTCTGATTA
                                 ARS 309(82%)
4651 AAAAATACAACATAGAAATATAATATTACATATAGATATAAAGAATAGAA
     TTTTTATGTTGTATCTTTATATTATAATGTATATCTATATTTCTTATCTT

4701 TAATATGGTACTTACTTACTGTTAGAAATAATAGAACTTTTGGAATAACA
     ATTATACCATGAATGAATGTCAATCTTTATTATGTTGAAAACCTTATTGT
                          B2 (72%)
4751 AGATAGTTAGTTGGTTTATGTTATATAGAATATAAGAAGATGATGTACAA
     TCTATCAATCAACCAAATACAATATATCTTATATTCTTCTACTACATGTT
     ARS 301(75%)
4801 AGAATAAAAGGGTGGGAGGGTGGTTGGTTGGTACTCCCTTAGACTGAATG
     TCTTATTTTCCCACCCTCCCACCAACCAACCATGAGGGAATCTGACTTAC
                                          B1 (78%)
4851 TTAGGGACCAAAAAAATAATAAAATTCTTGAAAACCCAACAAGGACTACT
     AATCCCTGGTTTTTTTATTTTTATTTTAAGAACTTTTGGGTTGTTCCTGA

4901 GTCATATTCAGTGAACCAACTGAACCATTAGTATCAATATGATTTTAGGG
     CAGTATAAGTCACTTGGTTGACTTGGTAATCATAGTTATACTAAAATCCC

4951 TGGGGGGGTGGGAGATACATATGTTCACTATGGACCAACTGGTACTGGTT
     ACCCCCCCACCCTCTATGTATACAAGTGATACCTGGTTGACCATGACCAA

5001 GGTTGCTCTGCTCCAACCAACCAGACCGGCTCTGCCGGTCTGGTTGGTTG
     CCAACGAGACGAGGTTGGTTGGTCTGGCCGAGACGGCCAGACCAACCAAC

5051 AGCGCAACCAACCAGTACCAGTTGGTCCATAGTGAACATATGTATCTCCC
     TCGCGTTGGTTGGTCATGGTCAACCAGGTATCACTTGTATACATAGAGGG
                     B2 (73%)
5101 ACCCCCCCACCCTAAAAACATATTGATACTAATGG
     TGGGGGGGTGGGATTTTTGTATAACTATGATTACC
```

Figure 15. Sequence comparison between LuIII genome and the ARS elements described. The boxed sequence represents the LuIII A/T rich region at m.u. 89. Bold sequences represent sequences found in the LuIII genome which share homology with the ARS elements (ARS consensus, B1 and B2) characterized. This homology is expressed as a percentage.

If this event in fact were occur at the right terminus during LuIII replication, it is possible that the DNA unwinding function could serve to facilitate the entry of the helicase and the additional replication machinery that primes and initiates the synthesis of LuIII plus strand. It has been proposed that the encapsidation is not selective, and thus every strand that is produced is encapsidated. Since the A/T rich sequences are not found in the MVM right terminus and in virtue of the homology that the LuIII-right terminus shares with ARS elements characterized in yeast, it is possible that LuIII could be synthesizing more plus strand (50%) than MVM (1%). These observations are in agreement with the kinetic model of parvovirus replication proposed by Chen (17) that suggests that the rates of the hairpin transfer determines the DNA distributions seen among parvoviruses.

CHAPTER V
Conclusion

The recombinant clone pUra-Lu88-100 containing map unit 88-100 (nt. 4527-5135) of parvovirus LuIII was capable of transforming *Saccharomyces cerevisiae* (SEY-2108, ura3-). Restriction and Southern Blot Analyses confirmed the recovery pUra-Lu88-100 from yeast (SEY-2108, ura3-). This data suggest that the A/T rich region of LuIII alone cannot act as an efficient autonomously replicating sequence; this region requires additional LuIII sequences present in the right terminus (m.u. 88-100) to enhance the observed ARS function in yeast.

CHAPTER VI

Recommendations

1. Deletions of internal segments and linker substitutions to identify functional regions required for autonomous replication present at map units 88-100 of LuIII, and examine their effects on ARS function.
2. Clone LuIII (m.u. 88-100) sequences into a plasmid that contain a CEN element to determine the plasmid stability.
3. Analysis of growth between the positive control (pRGM) and the experimental (pUra-Lu88-100).
4. Insert LuIII (m.u. 88-100) sequences into an expression vector and transfect human cells to test a possible origin of replication in higher eukaryotic cells.

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